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Enhanced chloroplastic generation of H_2O_2 in stress-resistant *Thellungiella salsuginea* in comparison to *Arabidopsis thaliana*

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In order to find some basis of salinity resistance in the chloroplastic metabolism, a halophytic *Thellungiella salsuginea* was compared with glycophytic *Arabidopsis thaliana*. In control *T.s.* plants the increased ratios of chlorophyll *a*/*b* and of fluorescence emission at 77 K (F_{730}/F_{685}) were documented, in comparison to *A.t.*. This was accompanied by a higher Y_{II} and lower NPQ (non-photochemical quenching) values, and by a more active PSI (photosystem I). Another prominent feature of the photosynthetic electron transport (PET) in *T.s.* was the intensive production of H_2O_2 from PQ (plastoquinone) pool. Salinity treatment (0.15 and 0.30 *M* NaCl for *A.t.* and *T.s.*, respectively) led to a decrease in ratios of chl *a*/*b* and F_{730}/F_{685}. In *A.t.*, a salinity-driven enhancement of Y_{II} and NPQ was found, in association with the stimulation of H_2O_2 production from PQ pool. In contrast, in salinity-treated *T.s.*, these variables were similar as in controls. The intensive H_2O_2 generation was accompanied by a high activity of PTOX (plastid terminal oxidase), whilst inhibition of this enzyme led to an increased H_2O_2 formation. It is hypothesized, that the intensive H_2O_2 generation from PQ pool might be an important element of stress preparedness in *Thellungiella* plants. In control *T.s.* plants, a higher activation state of carboxylase ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) was also documented in concert with the attachment of Rubisco activase (RCA) to the thylakoid membranes. It is supposed, that a closer contact of RCA with PSI in *T.s.* enables a more efficient Rubisco activation than in *A.t.*

Introduction

Plants' reaction to salinity is very complex, as visualized by recognition of 2171 salt-responsive proteins (Zhang et al. 2012). There is much evidence that halophytes are using the similar metabolic pathways as glycophytes to cope with salinity but at different transcription control (Dassanayake et al. 2011). Such a multiplicity of stress responses is not surprising considering a broad range of responses associated with: (1) stress signaling, (2) scavenging of reactive oxygen species (ROS), (3) protection against ion toxicity, (4) osmotic adjustment, (5) repair,

Abbreviations – AsA, ascorbic acid; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenolindophenol; DNP-INT, dinitrophenylether of 2-iodo-4-nitrothymol; LHC, light harvesting-complex; MV, methyl viologen; NPQ, non-photochemical quenching; OG, octyl gallate; PET, photosynthetic electron transport; PQ, plastoquinone; PQH_2, plastohydroquinone; PSI, photosystem I; PSII, photosystem II; PTOX, plastid terminal oxidase; PVPP, poly(vinylpolypyrrolidone); RCA, Rubisco activase; ROS, reactive oxygen species; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase.

(6) redirection of cell metabolism, (7) acceleration of development, etc. (for review refer Zhu 2001, Wang et al. 2003, Tuteja 2007, Munns and Tester 2008).

Much less is known about the protective adaptations of chloroplastic metabolism, however, apparently halophytes are capable for the efficient regulation of photosynthesis under stress. An example of such adaptation is the salinity-evoked C3-Crassulacean acid metabolism shift in the halophytic *Mesembryanthemum crystallinum*. Some protective chloroplastic strategies are exclusively stimulated by salinity in this halophytic species, among them malate valve (Gawronska et al. 2013). A comparison of closely related halophytic and glycophytic species seems to be a promising approach to identify crucial metabolic traits for salinity resistance. The two species from Brassicaceae family, *Arabidopsis thaliana* (a glycophyte) and *Thellungiella salsuginea* (former *halophila*, salt cress, a halophyte), recently emerged as a model couple in this respect (Inan et al. 2004, M'rah et al. 2006, Amtmann 2009, Stepien and Johnson 2009, Wu et al. 2012). Salt cress does not reveal any changes in morphology in comparison to *Arabidopsis*, except for smaller leaves, and its high resistance is profoundly based on biochemical and physiological adaptations. Several stress-related genes, like P5CS (putative Δ-1-pyrroline-5-carboxylate synthetase, enzyme of proline synthesis), FeSOD (iron superoxide dismutase) or SOS1 (Na^+/H^+ antiporter), are constitutively over-expressed in this species (Taji et al. 2004). These genes are representative for the three main strategies of salinity resistance described above. Due to the fact that this species is also resistant to other stresses (cold, drought) one may expect that some basis of stress resistance is also associated with the chloroplastic metabolism. Indeed, full-scale proteomic studies have shown that the majority of proteins affected by salt stress are affiliated with protein synthesis and photosynthesis (Pang et al. 2010).

Halophytic species typically grow at high irradiance, which might cover high energy cost of stress protection. This was shown in halophytic *M. crystallinum* plants which were not able to cope with high salinity below the threshold light intensity (Miszalski et al. 2001). Also, a higher light demand has been emphasized for halophytic *Thellungiella* in comparison to glycophytic *Arabidopsis* plants (Amtmann 2009). As demonstrated by Stepien and Johnson (2009), in *Thellungiella* plants a protective chlororespiratory pathway is activated under salinity. This pathway is associated with dissipation of proton gradient, which may, at least in part, explain increased light demand of this species. However, the increased light demand of *Thellungiella* in comparison to *Arabidopsis* already without salinity suggests the presence of some other pre-adaptive features of photosynthetic electron transport (PET). This work was undertaken to find differences in the chloroplastic metabolism between *Arabidopsis* and *Thellungiella*, which may create a basis for a high stress resistance of the halophytic species.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana (ecotype Columbia) and *T. salsuginea* (earlier *Thellungiella halophila*) ecotype Shandong plants were grown from seeds in the soil culture under irrigation with tap water. Plants were cultivated in the phytotron chamber at temperatures of 18/16°C day/night, photoperiod 10/14 h, irradiance of about 220 µmol m^{-2} s^{-1} and RH approximately 50%. Both species were adapted to these conditions for at least three generations. After development of leaf rosette, 4-week-old *A.t.* and *T.s.* plants were split into two groups: the first was further irrigated with water (controls), while the second with NaCl solution (0.15 *M* for *A.t.* and 0.3 *M* for *T.s.* were used). After 7th day of treatment the non-invasive measurements were done. For biochemical analysis leaves were harvested in the afternoon and frozen at −80°C until further use.

Determination of chlorophyll and relative antenna size

Chlorophyll was extracted from leaf powder (ab 0.1 g) with 1.6 ml of 80% acetone with addition of $MgCl_2$. Samples were mixed in closed and darkened Eppendorf tubes for 1 h. Concentration of chlorophyll was determined according to Lichtenthaler and Buschmann (2001). Frozen leaf powder was diluted in 0.05 *M* Hepes buffer pH 7.5, containing 0.330 *M* sorbitol. Low temperature chlorophyll fluorescence emission spectra were recorded using luminescence spectrometer LS50B (Perkin Elmer, Waltham, MA) at excitation of 437 nm.

Measurements of PSII efficiency

Parameters of PSII (photosystem II) efficiency were determined by the non-invasive fluorescence measurements with PAM 2500 (Heinz Walz GmbH, Effeltrich, Germany). Light curves were determined on plants adapted to the growth light conditions. A red actinic irradiation was used and each light intensity was applied for 3 min. The effective photochemical quantum yield of PSII (Y_{II}) was calculated according to Genty et al. (1989). Non-photochemical quenching (NPQ) of PSII fluorescence was quantified according to Kramer et al. (2004).

Isolation of thylakoids

Thylakoids were isolated essentially according to the modified methods of Casazza et al. (2001) and Suorsa et al. (2006). Leaves were mixed with ice-cold buffer containing 50 m*M* HEPES-KOH, pH 7.6, 330 m*M* sorbitol (control plants) and 495 m*M* sorbitol (salt-treated plants), 1 m*M* $MgCl_2$, 2 m*M* Na_2EDTA, 5 m*M* sodium ascorbate and 0,01% (w/v) fatty acids-free bovine serum albumin and then rapidly homogenized three times for 5 s. The homogenates were filtered through Miracloth and centrifuged at 4000 *g* for 4 min. The pellets were suspended in 50 m*M* HEPES-KOH, pH 7.6, 5 m*M* sorbitol, 5 m*M* $MgCl_2$ and centrifuged at 4000 *g* for 5 min. The pellets were washed with 50 m*M* Hepes-KOH, pH 7.6, 330 m*M* sorbitol, 10 m*M* $MgCl_2$, 20 m*M* NaCl, 2.5 m*M* EDTA, 10 m*M* $NaHCO_3$ and centrifuged at 4000 *g* for 5 min. Then the pellets were suspended in a small amount (2 ml) of the same buffer and were protected from light and kept ice-cold during the isolation procedure.

Measurements of PSI activity with oxygen electrode

Photochemical activity of isolated thylakoids was determined according to Hipkins and Baker (1986). Thylakoids (20 μg of chlorophyll) were re-suspended in 1 ml of the reaction medium that was composed of 0.05 *M* Hepes pH 7.6, containing 0.1 *M* sorbitol, 5 m*M* $MgCl_2$, 5 m*M* NaCl and in the presence of 5 m*M* sodium azide to inhibit peroxidase activity. The light-dependent oxygen consumption was followed by oxygen electrode (Oxytherm, Hansatech, UK) for ab 2 min at 25°C. Light intensity was 400 μmol m^{-2} s^{-1} at the surface of the measuring cell. Photosystem I (PSI)-specific electron transport was measured in the presence of 0.1 m*M* 2,6-dichlorophenolindophenol (DCPIP) and 5 m*M* ascorbic acid (AsA) as electron donors, and 100 μ*M* methyl viologen (MV) as an electron acceptor. In order to block electron transport from PSII 20 μ*M* 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was used.

Measurements of hydrogen peroxide

Production of hydrogen peroxide by isolated thylakoids was determined with Apollo 4000 Free Radical Analyzer (World Precision Instruments, Sarasota, FL) equipped with hydrogen peroxide sensor (ISO-HPO-2). Calibration of electrode was done by application of known H_2O_2 concentrations to the same medium as used for determination of the photochemical activity of thylakoids. No artificial donors and acceptors were added to the reaction media (a whole chain PET). Thylakoids (20 μg of chlorophyll) were re-suspended in 1 ml of the reaction medium that was composed of 0.05 *M* Hepes pH 7.6, containing 0.1 *M* sorbitol, 5 m*M* $MgCl_2$, 5 m*M* NaCl and in the presence of 5 m*M* sodium azide. The light-dependent hydrogen peroxide production was monitored for 2–3 min at 25°C. Light intensity was 400 μmol m^{-2} s^{-1} at the surface of the measuring cell. In order to block electron transport from PSII 20 μ*M* DCMU was used. Plastoquinol oxidation by cyt b_6f complex was blocked by 35 μ*M* DNP-INT (dinitrophenylether of 2-iodo-4-nitrothymol). The activity of plastid terminal oxidase (PTOX) was suppressed by 2 μ*M* octyl gallate (OG).

Rubisco activity

For measurements of activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39), leaf soluble proteins were extracted in 0.05 *M* Hepes-KOH buffer pH 8.0 containing, 10 m*M* *β*-mercaptoethanol, 15% glycerol, 2% poly(vinylpolypyrrolidone) (PVPP), 5 m*M* EDTA, 10 m*M* $MgCl_2$ and protease inhibitor cocktail (ProteoBlock™; Fermentas, Waltham, MA). Homogenates were centrifuged for 5 min at 13 300 *g* and supernatant was used for measurements. Activity of Rubisco was measured as described by Gubernator et al. (2008), by measuring the incorporation of ^{14}C ($NaH^{14}CO_3$) into acid-stable products which were determined using a liquid scintillator counter. The comparison of initial and total activity represents the fraction of the Rubisco activity which is dependent upon activation by carbon dioxide and Mg^{2+}.

SDS–PAGE and immunoblot analysis

For immunoblotting leaf soluble proteins were extracted in buffer composed of 0.1 *M* phosphate buffer pH 7.5, containing 5 m*M* EDTA, 1 m*M* dithiothreitol, 2% PVPP and protease inhibitor cocktail (ProteoBlock). Homogenates were centrifuged for 5 min at 10 000 *g* and supernatant was used for measurements. Soluble proteins and thylakoid membranes were dissolved in denaturing buffer (1:1, v:v), and SDS–PAGE and immunoblotting were done as described previously by Niewiadomska et al. (2009). Protein separation was performed on 12% polyacrylamide gels. All antibodies were obtained from Agrisera (Vännäs, Sweden).

Statistical analysis

All analysis were done with Student's *t*-test. Significances are marked at $P < 0.05$.

Results

Leaves of control *Arabidopsis* and *Thellungiella* plants were characterized by a similar chlorophyll *a*+*b* content amounting to 1.02 ± 0.05 and 1.10 ± 0.04 μg mg^{-1} FW in *A.t.* and *T.s.*, respectively. However, the composition of chl was slightly different, as demonstrated by a higher chl *a*/*b* ratio in *T.s.* amounting to 2.92, in comparison to 2.52 in *A.t.* (Fig. 1A). After the salinity-treatment amounts of chlorophyll were 1.1 ± 0.04 and 1.3 ± 0.07 in *A.t.* and *T.s.*, respectively, while, the chl *a*/*b* ratio declined in both species to 2.31 and 2.2, in *A.t.* and *T.s.*, respectively. To get a further insight into the relative sizes of PSI and PSII antennae, we monitored the chlorophyll fluorescence emission at 77 K. In this method, PSI- and PSII-associated chlorophyll antennae are reflected by the emission peaks at 730 and 685 nm, respectively. In control conditions, the proportion of chlorophyll associated with PSI in *T.s.* was significantly higher than in *A.t.* (Fig. 1B). A bigger contribution of PSI antenna may result from a permanent change in the thylakoid architecture or from a temporary migration of part of LHCII (light harvesting-complex II) antennae to PSI (state 1 to state 2 transition) driven by a reduced plastoquinone (PQ) pool. To resolve that, we also analyzed changes in the F_{730}/F_{685} during night–day transition, however, no differences between the two species were detected (data not shown).

PSII performance in the light-adapted state (Y_{II}) was monitored at increasing intensities of actinic light (Fig. 2A). In *T.s.*, Y_{II} values were significantly higher than in *A.t.* (significances start from the light intensity of 23 μmol m^{-2} s^{-1}). Salinity treatment did not change Y_{II} in *T.s.*, whereas, a significant increase was noted in *A.t.*, with exception of the highest light intensity used. However, Y_{II} values in salt-treated *T.s.* were still higher than in salt-treated *A.t.* (significances were found in the range of light intensities between 23 and 900 μmol m^{-2} s^{-1}). In the control *T.s.* plants, values of NPQ were lower than in *A.t.* in the range of light intensities from 185 to 455 μmol m^{-2} s^{-1} (Fig. 2B). A difference in NPQ between these two species became more pronounced after salinity treatment (significances from 185 to 900 μmol m^{-2} s^{-1}).

Changes in PSI activity were detected by mean of oxygen consumption by isolated thylakoids supplied with electron donors (DCPiP and AsA) and acceptor (MV) at light intensity of 400 μmol quanta m^{-2} s^{-1} (Fig. 3). This light intensity was chosen after checking the light dependence of PSI activity (Fig. S1) in order to maximize PET and, at the same time, to avoid photoinhibition. In thylakoids isolated from control *Thellungiella* plants activity of PSI was 27% higher than in thylakoids from *Arabidopsis*. Salinity treatment strongly decreased PSI activity in *Arabidopsis* (by about 50%), while in *T.s.* only a slight reduction (by 7%) was noted.

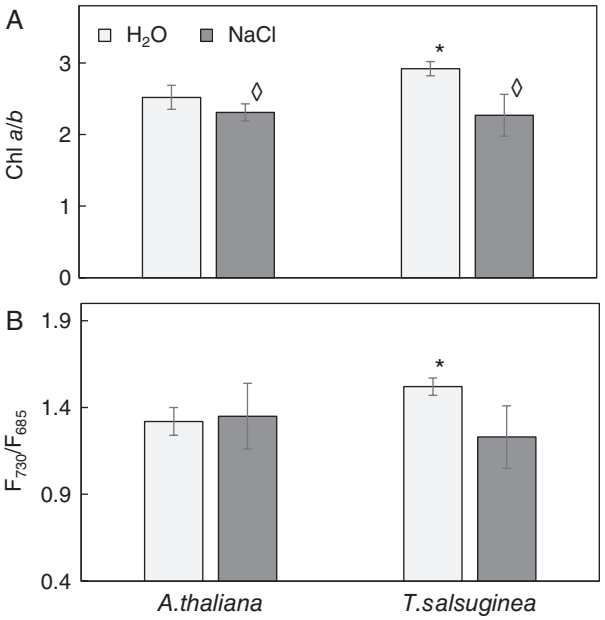

Fig. 1. The ratio of chlorophyll *a*/*b* ratio (A) and the ratio of chlorophyll fluorescence emission at 730/685 nm determined at 77 K (B) in leaves of *Arabidopsis thaliana* and *Thellungiella salsuginea* plants irrigated with water and with NaCl solution. Salinity of 0.15 and 0.3 *M* NaCl was used for *A.t.* and *T.s.*, respectively. Data represent mean ± SD (n = 6 and n = 3 for A and B, respectively). 'Diamond' indicates a significant difference between control and salinity-treated plants. 'Asterisk' indicates a significant difference from *A.t.* from the same treatment.

To compare a whole chain electron transport in the two species we followed the H_2O_2 production by illuminated thylakoids in the absence of artificial electron donors and acceptors. This approach was chosen instead of the typical method, based on the rate of O_2 uptake, to avoid a possible error which might be caused by the oxygen-consuming activity of PTOX, because literature data (Stepien and Johnson 2009) points to the increased participation of chlororespiration in *T.s.* Thylakoids isolated from control *T.s.* plants were characterized by about 2.3 times more intensive H_2O_2 production, in comparison to *A.t.* (Fig. 4). After salinity treatment an enhancement of H_2O_2 production was noted in both species, however it was significant only in *A.t.*

To check the origin of H_2O_2 formation we use several inhibitors of PET. The typical runs from the electrode –/+ inhibitors are shown in Fig. S2. In the presence of DCMU, production of H_2O_2 by isolated thylakoids from control *A.t.* and *T.s.* plants was totally suppressed, indicating that this ROS formation undergo at the expense of electrons from PSII (Fig. 5A). To verify whether H_2O_2 production take place at PSI, electron transport was

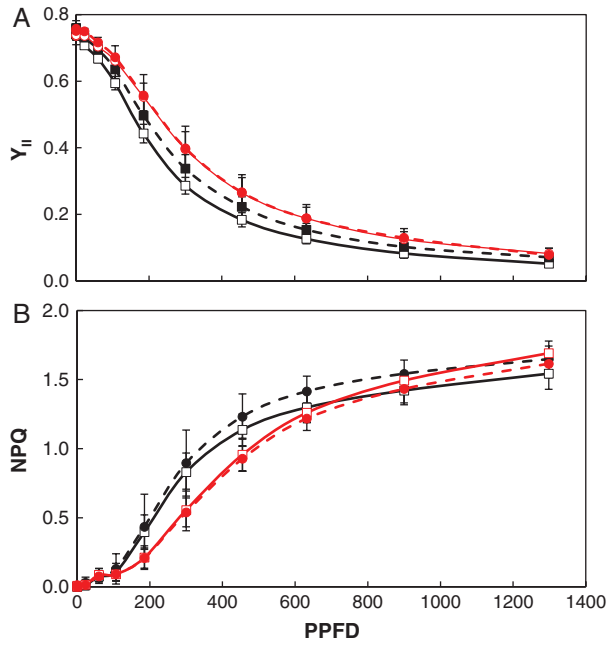

Fig. 2. Light curves of Y_{II} (A) and NPQ (B) in leaves of *Arabidopsis thaliana* (black lines, squares) and *Thellungiella salsuginea* (red lines, circles) plants irrigated with water (solid lines, empty symbols) and with NaCl solution (dashed lines, filled symbols). Salinity of 0.15 and 0.3 *M* NaCl was used for *A.t.* and *T.s.*, respectively. Values represent mean (n = 10). For significances see description in section Results.

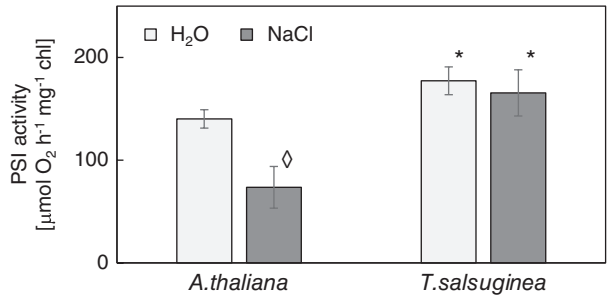

Fig. 3. A comparison of PSI activities measured with oxygen electrode (A) and with H_2O_2 electrode (B). Thylakoids were isolated from leaves of *Arabidopsis thaliana* and *Thellungiella salsuginea* plants irrigated with water and with NaCl solution. Salinity of 0.15 and 0.3 *M* NaCl was used for *A.t.* and *T.s.*, respectively. Data represent mean ± SD (n = 4). 'Diamond' indicates a significant difference between control and salinity-treated plants. 'Asterisk' indicates a significant difference from *A.t.* from the same treatment.

inhibited at the level of cyt b_6f by the addition of DNP-INT. This compound inhibited gross of H_2O_2 production in *Arabidopsis* (90%) suggesting that Mehler reaction might be a major place of its formation. In contrast, the application of this compound slightly stimulated the H_2O_2 production in *Thellungiella* (by 21%). On this basis, we concluded that in *T.s.* H_2O_2 is

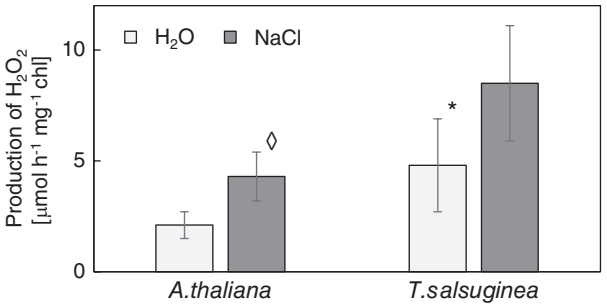

Fig. 4. Rates of H_2O_2 production by illuminated thylakoids from *Arabidopsis thaliana* and *Thellungiella salsuginea* plants irrigated with water or with NaCl solution. Salinity of 0.15 and 0.3 *M* NaCl was used for *A.t.* and *T.s.*, respectively. Data represent mean ± SD (n ≥ 5). 'Diamond' indicates a significant difference between control and salinity-treated plants. 'Asterisk' indicates a significant difference from *A.t.* from the same treatment.

formed predominantly at PQ pool. The addition of PTOX inhibitor OG (Josse et al. 2003) caused a significant stimulation of H_2O_2 formation (by 47%) in *T.s.* This result visualizes PTOX activity in the control *T.s.* plants and, at the same time, points to its protective function against ROS formation. On the other hand, a slight decrease in H_2O_2 formation (by 17%) caused by OG in *A.t.* in the absence of chlororespiration might be explained by a partial quenching of chlorophyll excitation, as this compound is the antioxidant and quencher of triplet states. If so, it is reasonable to assume that a partial quenching of PSII excitation by OG takes place also in *T.s.*, hence a net contribution of chlororespiration would be slightly higher.

As a next step, we checked whether any change in the place of ROS formation occurs due to salinity (Fig. 5B). After salinity treatment, H_2O_2 production from illuminated thylakoids of both species became slightly less sensitive to DCMU. The resting H_2O_2 generation was similar in *A.t.* and *T.s.* and amounted to about 26 and 28%, respectively. Since, this inhibitor blocks the electron transfer from Q_A to Q_B, this suggest that salinity stress may cause some alteration in the place and mechanism of H_2O_2 production toward a possible place before $Q_A^{\bullet-}$. As reviewed by Pospíšil (2012), H_2O_2 may also arise from the donor side of PSII. In *Arabidopsis*, a much lower inhibitory effect was imposed by DNP-INT (inhibition of H_2O_2 formation only by 13%). This suggests that participation of Mehler reaction in the production of ROS was strongly reduced. In *Thellungiella*, a 22% stimulation was noted due to DNP-INT, similarly to the value detected in controls. The addition of OG caused a stimulation of H_2O_2 production in *Arabidopsis* and *Thellungiella* by 29 and 57%, respectively, indicating a stimulation of chlororespiration in both

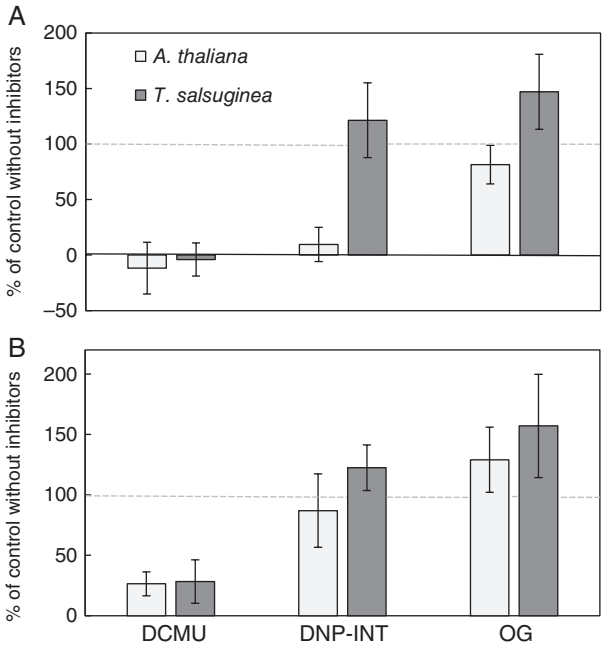

Fig. 5. Effects of inhibitors on the rate of H_2O_2 production by illuminated thylakoids from *Arabidopsis thaliana* and *Thellungiella salsuginea* plants irrigated with water (A) or with NaCl solution (B), as in Fig. 4. Data represent mean ± SD (n ≥ 4). Inhibitors were added during the run after ab 2 min of irradiance, and rate of H_2O_2 production was monitored for another 2 min. The final concentration of DCMU was 20 μ*M*, DNP-INT 35 μ*M* and OG 2 μ*M*.

species. It is to be noted that thylakoids isolated from the control *A.t.* plants were coupled to the proton gradient by 45.7%, in comparison to 36% in *T.s.* (Fig. S3). After salinity treatment the coupling state was reduced in *A.t.* (22.7%), and only slightly in *T.s.* (32.7%).

The constitutive activity of PTOX, uncoupling of thylakoids and the intensive H_2O_2 formation, already in control *Thellungiella* plants, rises a question: Whether it could impede a process of carbon assimilation? To verify that, we compared the amounts of proteins at the PSI acceptor side: Rubisco activase (RCA) and Rubisco. A 33 kDa subunit of oxygen evolving complex (PsbO) was used as a reference. Because a growing body of data documented a dual localization of RCA, soluble in the stroma and attached to the thylakoid membranes (Eichelmann et al. 2009, Chen et al. 2010), we looked for these proteins in both of these locations. In leaf extracts as in the thylakoid membranes the level of PSII protein PsbO was similar in both species (Fig. 6). In contrast, the two RCA bands (42 and 47 kDa) were more abundant in leaf extracts from *T.s.* than in *A.t.*, and difference between the two species became more pronounced when the fraction of TM was considered. This indicates that in *T.s.* more RCA is attached to the thylakoid membranes. The total level of Rubisco (rbcL 53 kDa band) was similar in *A.t.* and *T.s.*, however, in *T.s.* a bigger portion of Rubisco was present in the thylakoid membrane fraction. We suppose that this represents a part of Rubisco currently in contact with a membrane-bound RCA. The initial and total activity of Rubisco was significantly higher in *T.s.* in comparison to *A.t.* (Fig. 7). Hence, either a higher activation state of Rubisco or a low level of Rubisco inhibitors might be concluded in this species in comparison to *A.t.*

Discussion

Salinity causes oxidative stress and disturbs the basic metabolic fluxes in mesophyll cells (Tuteja 2007, Miller et al. 2010). As several harmful effects of salinity are exerted on chloroplastic metabolism, we hypothesize that specific adaptations of PET may be a pre-requisite for a high salinity resistance. To verify that we compared the performance of PET in halophytic *T.s.* plant with the closely related glycophytic species *A.t.*

In this article we documented a higher ratio of chl *a*/*b* in leaves of the control *Thellungiella* plants in comparison to leaves of *Arabidopsis*. Since chlorophyll *b* is present mainly in the LHC of granal thylakoids (Anderson and Andersson 1982, Melis 1991), its relative decline in comparison to chl *a* may indicate a smaller granal stacking and higher relative contribution of PSI. This conclusion is supported by a higher amount of PSI-associated antennae in proportion to those associated with PSII, as reflected by F_{730}/F_{685} ratio, and by a higher PSI activity in *T.s.* revealed with isolated thylakoids. A salinity-driven decline in chl *a*/*b* ratios was a common feature of both species. A similar effect was previously described for the halophytic *Bruguiera parviflora* (Parida and Das 2005), and most likely represents a reduction of the antennae size. However, in *T.s.*, a stronger reduction of PSI-associated antennae in comparison to those associated with PSII might be concluded from F_{730}/F_{685}.

Functioning of PET in *Thellungiella* was also characterized by a higher efficiency of PSII, in comparison to *Arabidopsis*. The enhanced Y_{II} in *T.s.* has previously been demonstrated by Stepien and Johnson (2009), however, only in salinity-treated plants subjected to a short-term high irradiance. This effect was explained by the induction of protective cyclic electron flow around PSII. Higher Y_{II} values already in control *T.s.* plants, observed in our study, may result from the high intensity of growth light (220 μmol m^{-2} s^{-1}) in comparison to 120 μmol m^{-2} s^{-1} used before. Also, in contrast to earlier study, an intensification of Y_{II} was noted in *A.t.* after the salinity treatment. This was associated by increased NPQ, which informs about excessive excitation pressure at PSII associated with increased dissipation of captured energy as

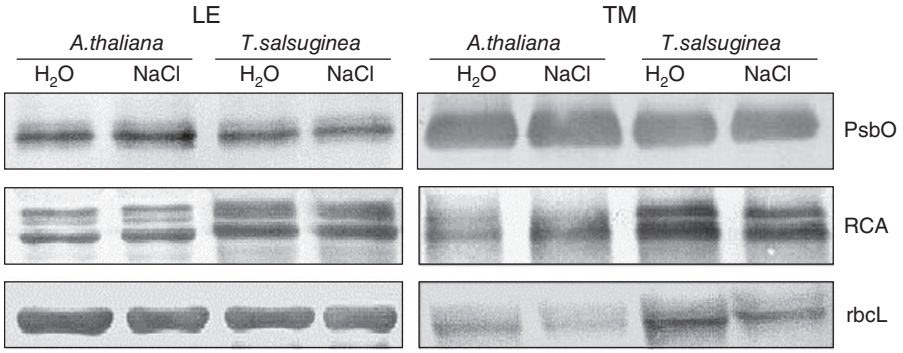

Fig. 6. Changes in the amount of PsbO, RCA and large subunit of Rubisco (rbcL) in *Arabidopsis thaliana* and *Thellungiella salsuginea* plants irrigated with water and with NaCl solution (0.15 and 0.3 *M* NaCl, respectively). Immunoblotting was done with leaf extracts (LE) and with the thylakoid membranes (TM). For visualization of PsbO, RCA and rbcL in LE: 10, 10 and 5 μg protein per lane were loaded, respectively. For visualization of PsbO, RCA and rbcL in TM: 2, 5 and 8 μg chlorophyll per lane were loaded, respectively. Blots are representative for at least four repetitions.

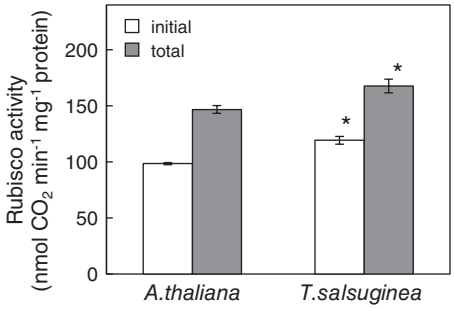

Fig. 7. The initial and total activity of Rubisco measured in leaves of *Arabidopsis thaliana* and *Thellungiella salsuginea* plants treated with water. Data represent mean ± SD (n = 3). 'Asterisk' indicates a significant difference from *A.t.* from the same treatment.

heat. Whilst, the low NPQ values in *T.s.* may indicate the efficient electron transport at PSII in both conditions.

In this study we provide evidence that electron transport in *T.s.*, already in control conditions, is accompanied by the intensive generation of H_2O_2. It is generally assumed that in the absence of electron donors and acceptors ROS are formed predominantly in so-called Mehler reaction at PSI. However, a growing body of data points to many other places of ROS production in association with PSII. On the donor side of PSII, the two-electron oxidation of water results in H_2O_2 formation (Ananyev et al. 1992, Hillier and Wydrzynski 1993, Arato et al. 2004, Clausen and Junge 2004, Pospíšil 2012). Whereas, on the acceptor side of PSII, the one-electron reduction of molecular oxygen takes place from pheophytin, tightly bound QA, loosely bound QB, free plastosemiquinone (PQ) and cytochrome b559 (Graan and Ort 1984, McCauley and Melis 1986, Khorobrykh and Ivanov 2002, Khorobrykh et al. 2004, Mubarakshina et al. 2006, Pospíšil 2012). This superoxide anion radical is further reduced either by another $O_2^{\bullet-}$ molecule or by plastohydroquinone (PQH_2) to free hydrogen peroxide. In *Thellungiella*, production from the donor side of PSII is excluded by DCMU effect, because this inhibitor blocks oxidation of Q_A, which in consequence would increase the excitation pressure at PSII, as well as, ROS formation. In contrast to *A.t.*, production of H_2O_2 in *T.s.* was not blocked by DNP-INT (inhibitor of cyt b6f complex) which indicates that this is predominantly formed at PQ pool. According to Khorobrykh and Ivanov (2002) and Khorobrykh et al. (2004), formation of H_2O_2 from PQ pool involves a reduction of superoxide by PQH_2, where superoxide may come from both PQ pool and PSI. It remains to be elucidated, whether such intensive H_2O_2 formation in *T.s.* does occur in vivo. As shown by Casano et al. (2000), H_2O_2 formed aside of PET might be rapidly scavenged by peroxidase which uses plastoquinol as an electron donor, or by other peroxidases, which use ascorbate or glutathione as electron donors. An intensive production and scavenging of H_2O_2 could account for a rapid cycling of electrons close to PSII, and may be a tempting alternative to a water–water cycle starting from PSI.

Chlororespiration might be another photoprotective strategy utilized under salinity. Our data indicate that PTOX activity was present already in control *T.s.* plants. This is different from the previous data described by Stepien and Johnson (2009), where stimulation of this enzyme was salinity-dependent. This discrepancy might be explained by a stronger stress at high irradiance or by a more sensitive method employed here. As presented here, chlororespiration seems to be correlated with the enhancement in Y_{II}, lower NPQ, as well as with the intensive H_2O_2 generation from PQ pool. However, by showing a partial limitation of H_2O_2 generation

whenever PTOX activity was present, our data are different from those obtained with PTOX-overexpressing *Arabidopsis* and tobacco plants, where no photoprotection was found (Joët et al. 2002, Rosso et al. 2006). Moreover, overexpression of PTOX in tobacco was associated by enhanced production of superoxide and hydroxyl radical by illuminated thylakoids (Heyno et al. 2009). Apparently, overexpression of PTOX is not sufficient to confer stress resistance under acute high irradiance. However, our data suggest that activation of PTOX might be a common phenomenon under a mild salinity stress. Further studies are necessary to define more precisely the conditions where PTOX activity is induced, and what is the relation between PTOX activity and H_2O_2 production by PET. It is noteworthy that activation of PTOX seems to be correlated with some thylakoid uncoupling. A lower coupling of thylakoids from a more stress-resistant *T.s.* in comparison to *A.t.*, already in control conditions, might be similar to the adaptation to chilling documented by Peeler and Naylor (1988). These authors found that thylakoids isolated from chilling-resistant species (*Pisum sativum* cv Alaska) were much less coupled than those from chilling-sensitive species (*Cucumis sativus* cv Ashley).

Several cues detected in *A.t.* after a mild salinity (enhanced Y_{II}, increased H_2O_2 generation from PQ pool, induction of PTOX and lower coupling state of isolated thylakoids) were present already in control *Thellungiella* plants. This may indicate that *T.s.* is pre-adapted to stress, in agreement with several earlier studies (Inan et al. 2004, Taji et al. 2004, Gong et al. 2005, Kant et al. 2006, Amtmann 2009). Hence, we postulate that a high H_2O_2 production is an important element of this pre-adaptation. The intensive H_2O_2 generation may keep antioxidant system in the activated state and serve as a signal for gene expression. It remains, however, unresolved what mechanism ensures a high H_2O_2 generation in *T.s.* in the absence of stress. It might be speculated that an undefined defect at the intersystem electron transport chain might be responsible for this phenomenon.

As pointed out by Khorobrykh et al. (2004) and Mubarakshina et al. (2006), production of H_2O_2 at PQ pool is directed to the thylakoid lumen. This leads to a decrease in proton gradient. Hence, it is strongly suggested that photosynthesis in *T.s.* might be ATP-limited already in the control conditions. This situation would restrict the assimilation of CO_2 in Calvin cycle. In agreement with that supposition, we revealed similar amounts of Rubisco and psbO, but higher abundance of RCA in *T.s.* in comparison to *A.t*. Moreover, a majority of RCA in *T.s.* was attached to the thylakoid membranes. Such a localization of RCA has been evidenced before. Eichelmann et al. (2009) suggested that a temporary attachment of activase to the thylakoid membranes (close to cyt b_6f) is necessary for the restoration of its active form in close correlation with PSI activity. Whereas Chen et al. (2010) indicated that attachment of RCA is correlated with the low pH gradient across the thylakoid membrane, as well as with the low ATP production. These data support the hypothesis of a low pH gradient and low ATP production in chloroplast of *T.s.* In view of this, the enhanced Rubisco activation demonstrated here may represent a compensatory mechanism for the low ATP supply.

In conclusion, this study reports on the intensive high production of hydrogen peroxide from PQ pool by thylakoids from halophytic *Thellungiella*, which seems to be associated with the anticipation of stress in this highly stress-resistant species. This phenomenon is associated with the intensive chlororespiration and attachment of RCA to the thylakoid membranes which seems to ensure a high activation state of Rubisco at low proton gradient.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Activity of PSI in relation to light intensity in thylakoids isolated from leaves of *Arabidopsis thaliana* and *Thellungiella salsuginea* plants.

Fig. S2. Effects of irradiance and inhibitors on the H_2O_2 production by isolated thylakoids from leaves of *Arabidopsis thaliana* (A) and *Thellungiella salsuginea* (B). Measurements started in the dark, starting points of the irradiances of 400 and 800 μmol m^{-2} s^{-1} and cutoff the light, are marked. The typical runs for control thylakoids (blue), and for thylakoids incubated with DCMU (red), DNP-INT (gray) and OG (yellow) are shown. The composition of the thylakoid medium and concentrations of inhibitors are as described in section Materials and methods. The amount of 20 μg of chlorophyll *a*+*b* was loaded to the 1 ml reaction vial.

Fig. S3. Coupling state of thylakoids isolated from leaves of *Arabidopsis thaliana* and *Thellungiella salsuginea*. PSII-mediated electron transport rates were determined spectrophotometrically by measuring photoreduction of DCPIP (μmol of reduced DCPIP h^{-1} mg chl^{-1}) at 590 nm, in the absence and presence of uncoupler (10 m*M* NH_4Cl). The reaction mixture was composed of thylakoids (10 μg Chl ml^{-1}) suspended in the medium, as described in section Materials and methods, and of 35 μ*M* DCPIP. Calculated coupling states are given in brackets. Data represent mean ± SD (n = 3–4).

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